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(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) TNF Peptides

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NOVEL TNF PEPTIDES

The present invention relates to novel peptides derived from tumor necrosis factor (TNF), the preparation thereof and the use thereof as drugs.

Carswell et al. (Proc. Natl. Acad. Sci. USA 72 (1975) 3666) reported that the serum of endotoxin-treated animals which had previously been infected with the Calmette-Guerin strain of Mycobacteria (BCG) brought about hemorrhagic necrosis in various mouse tumors. This activity was ascribed to tumor necrosis factor. TNF also has a cytostatic or cytotoxic effect on a large number of transformed cell lines in vitro, whereas normal human and animal cell lines are unaffected (Lymphokine Reports Vol. 2, pp 235-275, Academic Press, New York, 1981). Recently, the biochemical characterization and the gene for human TNF have been described (Nature 312 (1984) 724, J. Biol. Chem. 260 (1985) 2345, Nucl. Acids Res. 13 (1985) 6361).

It is possible to deduce from this data the following protein structure for mature human TNF:

ValArgSerSerSerArgThrProSerAspLysProValAlaHisValValAlaAsnPro
GlnAlaGluGlyGlnLeuGlnTrpLeuAsnArgArgAlaAsnAlaLeuLeuAlaAsnGly
ValGluLeuArgAspAsnGlnLeuValValProSerGluGlyLeuTyrLeuIleTyrSer
GlnValLeuPheLysGlyGlnGlyCysProSerThrHisValLeuLeuThrHisThrIle
SerArgIleAlaValSerTyrGlnThrLysValAsnLeuLeuSerAlaIleLysSerPro
CysGlnArgGluThrProGluGlyAlaGluAlaLysProTrpTyrGluProIleTyrLeu
GlyGlyValPheGlnLeuGluLysGlyAspArgLeuSerAlaGluIleAsnArgProAsp
TyrLeuAspPheAlaGluSerGlyGlnValTyrPheGlyIleIleAlaLeu

The TNF genes of cattle, rabbits and mice have also been described (Cold Spring Harbor Symp. Quant. Biol. 51 (1986) 597).

Besides its cytotoxic properties, TNF is one of the main

substances involved in inflammatory reactions (Pharmac. Res. 5 (1988) 129). Animal models have shown that TNF is involved in septic shock (Science 229 (1985) 869) and graft-versus-host disease (J. Exp. Med. 166 (1987) 1280).

We have now found that peptides with a considerably lower molecular weight have beneficial properties.

The present invention relates to peptides of the formula I

X-A-Gly-B-Y I

where

A is Asn, Asp or His,

B is Val, Met or Phe,

X is G-NH-CHM-CO-, G-NH-CHM-CO-W-, G-R-NH-CHM-CO- or G--R-NH-CHM-CO W- and

Y is -Z,-NH-CHQ-CO-Z, -V-NH-CHQ-CO-Z, -NH-CHQ-CO-U-Z or -V-NH-CHQ-CO-U-Z

where, in X and Y,

G is hydrogen or an amino-protective group,

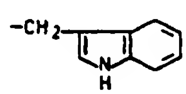
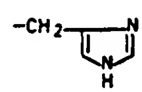
Z is OH or NH_2 or a carboxyl-protective group or

G and Z together are also a covalent bond or $-CO-(CH_2)_a-NH-$ where a is from 1 to 12,

R, U, V and W are peptide chains composed of 1-4 naturally occurring α-amino acids, and

M and Q are hydrogens or one of the following

$-CH(CH_3)_2$, $-CH(CH_3)-C_2H_5$, $-C_6H_5$, $-CH(OH)-CH_3$,

 ,  or $-(CH_2)_b-T$

(b being from 1 to 6 and T being hydrogen or OH, CH_3O, CH_3S, $(CH_3)_2CH$, C_6H_5, p-HO-C_6H_4, HS, H_2N, HO-CO, H_2N-CO, H_2N-C(=NH)-NH) or

M and Q together are a $-(CH_2)_c-S-S-(CH_2)_d-$, $-(CH_2)_e-CO-NH-(CH_2)_f$ or $-(CH_2)_e-NH-CO-(CH_2)_g-NH-CO-(CH_2)_f-$ bridge (with c and d being from 1 to 4, e and

f being from 1 to 6 and g being from 1 to 12),

as well as the salts thereof with physiologically tolerated acids.

The peptides of the formula I are constructed of L-amino acids, but they can contain 1 or 2 D-amino acids. The side-chains of the trifunctional amino acids can carry protective groups or be unprotected.

Particularly preferred physiologically tolerated acids are: hydrochloric acid, citric acid, tartaric acid, lactic acid, phosphoric acid, methanesulfonic acid, acetic acid, formic acid, maleic acid, fumaric acid, malic acid, succinic acid, malonic acid, sulfuric acid, L-glutamic acid, L-aspartic acid, pyruvic acid, mucic acid, benzoic acid, glucuronic acid, oxalic acid, ascorbic acid and acetylglycine.

The novel peptides can be open-chain (G = H, amino-protective group; Z = OH, NH_2, carboxyl-protective group, M and Q not connected together) and, in particular, have a disulfide bridge (G = H, amino-protective group; Z = OH, NH_2, carboxyl-protective group; M + Q = $-(CH_2)_c$-S-S-$(CH_2)_d$-) or a side chain bridge (G = H, amino-protective group, Z = OH, NH_2, carboxyl-protective group, M + Q = $-(CH_2)_e$-NH-CO-$(CH_2)_f$- or $-(CH_2)_e$-NH-CO-$(CH_2)_g$-NH-CO-$(CH_2)_f$-) or be linked head-to-tail (G + Z = covalent bond or -CO-$(CH_2)_a$-NH-).

The novel compounds can be prepared by conventional methods of peptide chemistry.

Thus, the peptides can be constructed sequentially from amino acids or by linking together suitable smaller peptide fragments. In the sequential construction, the peptide chain is extended stepwise, by one amino acid

each time, starting at the C terminus. In the case of coupling of fragments it is possible to link together fragments of different lengths, these in turn being obtainable by sequential construction from amino acids or coupling of other fragments. The cyclic peptides are obtained, after synthesis of the open-chain peptides, by a cyclization reaction carried out in high dilution.

In the case both of sequential construction and of fragment coupling it is necessary for the building blocks to be linked by formation of an amide linkage. Enzymatic and chemical methods are suitable for this.

Chemical methods for forming amide linkages are dealt with in detail by Müller, Methoden der Organischen Chemie (Methods of Organic Chemistry) Vol. XV/2, pp 1-364, Thieme Verlag, Stuttgart, 1974; Stewart, Young, Solid Phase Peptide Synthesis, pp 31-34, 71-82, Pierce Chemical Company, Rockford, 1984; Bodanszky, Klausner, Ondetti, Peptide Synthesis, pp 85-128, John Wiley & Sons, New York, 1976 and other standard works of peptide chemistry. Particularly preferred are the azide method, the symmetrical and mixed anhydride method, active esters generated in situ or preformed and the formation of amide linkages using coupling reagents (activators), in particular dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIC), 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), n-propanephosphonic anhydride (PPA), N,N-bis(2-oxo-3-oxazolidinyl)amidophosphoryl chloride (BOP-Cl), diphenylphosphoryl azide (DPPA), Castro's reagent (BOP), O-benzotriazolyl-N,N,N',N'-tetramethyluronium salts (HBTU), 2,5-diphenyl-2,3-dihydro-3-oxo-4-hydroxythiophene dioxide (Steglich's reagent; HOTDO) and 1,1'-carbonyldiimidazole (CDI). The coupling reagents can be employed alone or in combination with additives such as N,N'-dimethyl-4-aminopyridine (DMAP),

N-hydroxybenzotriazole (HOBt), N-hydroxybenzotriazine (HOOBt), N-hydroxysuccinimide (HOSu) or 2-hydroxypyridine.

Whereas it is normally possible to dispense with protective groups in enzymatic peptide synthesis, for chemical synthesis it is necessary for there to be reversible protection of the reactive functional groups which are not involved in the formation of the amide linkage on the two reactants. Three conventional protective group techniques are preferred for chemical peptide syntheses: the benzyloxycarbonyl (Z), the t-butyloxycarbonyl (Boc) and the 9-fluorenylmethyloxycarbonyl (Fmoc) techniques. In each case the protective group on the α-amino group of the chain-extending building block is identified. The side-chain protective groups on the trifunctional amino acids are chosen so that they are not necessarily eliminated together with the α-amino protective group. A detailed review of amino acid protective groups is given by Müller, Methoden der Organischen Chemie Vol XV/1, pp 20-906, Thieme Verlag, Stuttgart, 1974.

The building blocks used to construct the peptide chain can be reacted in solution, in suspension or by a method similar to that described by Merrifield in J. Amer. Chem. Soc. 85 (1963) 2149. Particularly preferred methods are those in which peptides are constructed sequentially or by fragment coupling by use of the Z, Boc or Fmoc protective group technique, in which case the reaction takes place in solution, as well as those in which, similar to the Merrifield technique, one reactant is bound to an insoluble polymeric support (also called resin hereinafter). This typically entails the peptide being constructed sequentially on the polymeric support, by use of the Boc or Fmoc protective group technique, with the growing peptide chain being covalently bonded at the C terminus to the insoluble resin particles (cf. Figures

1 and 2). This procedure allows reagents and byproducts to be removed by filtration, and thus recrystallization of intermediates is superfluous.

The protected amino acids can be bonded to any suitable polymers which merely need to be insoluble in the solvents used and to have a stable physical form which allows easy filtration. The polymer must contain a functional group to which the first protected amino acid can be firmly linked by a covalent bond. A wide variety of polymers is suitable for this purpose, for example cellulose, polyvinyl alcohol, polymethacrylate, sulfonated polystyrene, chloromethylated copolymer of styrene and divinylbenzene (Merrifield resin), 4-methylbenzhydrylamine-resin (MBHA-resin), phenylacetamidomethyl-resin (Pam-resin), p-benzyloxybenzyl alcohol-resin, benzhydrylamine-resin (BHA-resin), 4-hydroxymethyl-benzoyloxymethyl-resin, the resin used by Breipohl et al. (Tetrahedron Lett. 28 (1987) 565; from BACHEM), HYCRAM resin (from ORPEGEN) or SASRIN resin (from BACHEM).

Solvents suitable for peptide synthesis in solution are all those which are inert under the reaction conditions, in particular water, N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetonitrile, dichloromethane (DCM), 1,4-dioxane, tetrahydrofuran (THF), N-methyl-2-pyrrolidone (NMP) and mixtures of the said solvents. Peptide synthesis on polymeric supports can be carried out in all inert organic solvents which dissolve the amino acid derivatives used; however, solvents which also have resin-swelling properties are preferred, such as DMF, DCM, NMP, acetonitrile and DMSO, as well as mixtures of these solvents.

After the peptide has been synthesized it is cleaved off the polymeric support. The cleavage conditions for the various types of resins are disclosed in the literature.

The cleavage reactions most commonly use acid and palladium catalysis, in particular cleavage in anhydrous liquid hydrogen fluoride, in anhydrous trifluoromethanesulfonic acid, in dilute or concentrated trifluoroacetic acid or palladium catalyzed cleavage in THF or THF-DCM mixtures in the presence of a weak base such as morpholine. The protective groups may, depending on the choice thereof, be retained or likewise cleaved off under the cleavage conditions. Partial deprotection of the peptide may also be worthwhile if the intention is to carry out certain derivatization reactions or a cyclization.

Some of the novel peptides have good cytotoxic properties. Some others of the peptides have high affinity for the cellular TNF receptor without, however, having cytotoxic activity. They are therefore TNF antagonists. They compete with natural TNF for binding to the cellular TNF receptor and thus suppress the TNF effect. The novel peptides are valuable drugs which can be employed for treating neoplastic diseases and autoimmune diseases as well as for control-ling and preventing infections, inflammations and transplant rejection reactions. Simple experiments can be used to elucidate the mode of action of the individual peptides. The cytotoxicity of the peptide is determined by incubating a TNF-sensitive cell line in the presence of the peptide. In a second experimental approach, the cell line is incubated with the relevant peptide in the presence of a lethal amount of TNF. It is possible in this way to detect the TNF-antagonistic effect. In addition, the affinity of the peptide for the cellular TNF receptor is determined in an in vitro binding experiment.

The following test systems were used to characterize the agonistic and antagonistic effects of the novel peptides:

I. Cytotoxicity test on TNF-sensitive indicator cells,

II. Cytotoxicity antagonism test on TNF-sensitive indicator cells,

III. Competitive receptor-binding test on indicator cells expressing TNF receptor.

I. Cytotoxicity test

The agonistic effects of the novel peptides are assessed on the basis of their cytotoxic effect on TNF-sensitive cells (e.g. L929, MCF-7, A204, U937). The test with L929 and MCF-7 was carried out as follows:

1. 100 μl of culture medium containing 3 to 5 x 10^3 freshly trypsinized, exponentially growing, L929 cells (mouse) or MCF-7 cells (human) were pipetted into the wells of a 96-well flat-bottom culture plate. The plate was incubated at 37°C overnight. The air in the incubator was saturated with water vapor and contained 5% CO_2 by volume.

   The L929 culture medium contained 500 ml of 1x Earle's MEM (Boehringer Mannheim), 50 ml of heat-inactivated (56°C, 30 min) fetal calf serum (FCS), 50 ml of L-glutamine (200 mM), 5 ml of 100x non-essential amino acids, 3 ml of 1M HEPES buffer pH 7.2, and 50 ml of gentamicin (50 mg/ml).

   The MCF-7 culture medium contained 500 ml of 1x Dulbecco's MEM (Boehringer Mannheim), 100 ml of heat-inactivated (56°C, 30 min) FCS, 5 ml of L-glutamine and 5 ml of 100x non-essential amino acids.

2. The next day 100 μl of the peptide solution to be tested were added to the cell cultures and subjected to serial 2-fold dilution. In addition, some cell controls (i.e. cell cultures not

treated with peptide dilution) and some rhu-TNF controls (i.e. cell cultures treated with recombinant human TNF) were also made up. The culture plate was incubated at 37°C in an atmosphere of air saturated with water vapor and containing 5% CO_2 by volume for 48 h.

3. The percentage of surviving cells in the cultures treated with peptide dilution was determined by staining with crystal violet. For this purpose, the liquid was removed from the wells of the test plate by tapping it. 50 µl of crystal violet solution were pipetted into each well.

   The composition of the crystal violet solution was as follows:

   3.75 g of crystal violet
   1.75 g of NaCl
   161.5 ml of ethanol
   43.2 ml of 37% formaldehyde
   water ad 500 ml

   The crystal violet solution was left in the wells for 20 min and then likewise removed by tapping. The plates were then washed 5 times by immersion in water in order to remove dye not bound to the cells. The dye bound to the cells was extracted by adding 100 µl of reagent solution (50% ethanol, 0.1% glacial acetic acid, 49.9% water) to each well.

4. The plates were shaken for 5 min to obtain a solution of uniform color in each well. The surviving cells were determined by measuring the extinction at 540 nm of the colored solution in the individual wells.

5. Subsequently, by relating to the cell control, the 50% cytotoxicity value was defined, and the reciprocal of the sample dilution which resulted in 50% cytotoxicity was calculated as the cytotoxic activity of the test sample.

II. Cytotoxicity antagonism test

The antagonistic effect of the peptides was assessed on the basis of their property of antagonizing the cytotoxic effect of rhu-TNF on TNF-sensitive cells (e.g. L929, MCF-7, A204, U937). The cytotoxicity antagonism test with L929 and MCF-7 cells was carried out as follows:

1. 100 µl of culture medium containing 3 to 5 x 10^3 freshly trypsinized, exponentially growing, L929 cells (mouse) or MCF-7 cells (human) were pipetted into the wells of a 96-well flat-bottom culture plate. The plate was incubated at 37°C overnight. The air in the incubator was saturated with water vapor and contained 5% CO_2 by volume.

   The L929 culture medium contained 500 ml of 1x Earle's MEM (Boehringer Mannheim), 50 ml of heat-inactivated (56°C, 30 min) FCS, 5 ml of L-glutamine (200 mM), 5 ml of 100x non-essential amino acids, 3 ml of 1M HEPES buffer pH 7.2, and 500 µl of gentamicin (50 mg/ml).

   The MCF-7 culture medium contained 500 ml of 1x Dulbecco's MEM (Boehringer Mannheim), 100 ml of heat-inactivated (56°C, 30 min) FCS, 5 ml of L-glutamine (200 mM) and 5 ml of 100x non-essential amino acids.

2. The next day 100 µl of the peptide solution to be tested were added to the cell cultures and

subjected to serial 2-fold dilution. Then, 100 µl of a rhu-TNF dilution in culture medium, which dilution had an 80-100% cytotoxic effect in the final concentration in the cell culture, were added to these cell cultures. In addition, some cell controls (i.e. cell cultures not treated with peptide solution or with rhu-TNF solution) and some rhu-TNF controls (= cell cultures treated only with rhu-TNF solution) were also made up. The culture plate was then incubated at 37°C in an atmosphere of air saturated with water vapor and containing 5% CO_2 by volume for 48 h.

3. The percentage of surviving cells in the cultures treated with substance solution was determined by staining with crystal violet. For this purpose, the liquid was removed from the wells of the test plate by tapping it. 50 µl of crystal violet solution were pipetted into each well.

   The crystal violet solution had the composition specified in I.3

   The crystal violet solution was left in the wells for 20 min and then likewise removed by tapping. The plates were then washed 5 times by immersion in water in order to remove dye not bound to the cells. The dye bound to the cells was extracted by adding 100 µl of reagent solution (50% ethanol, 0.1% glacial acetic acid, 49.9% water) to each well.

4. The plates were shaken for 5 min to obtain a solution of uniform color in each well. The surviving cells were determined by measuring the extinction at 540 nm of the colored solution in the individual wells.

5. Subsequently, by relating to the cell control and the rhu-TNF control, the 50% antagonism value was defined, and the sample concentration which resulted in 50% antagonism of rhu-TNF cytotoxicity at the rhu-TNF concentration used was calculated as antagonistic activity of the sample tested.

III. Competitive receptor-binding test

Both the agonistic and antagonistic effects of peptides are conditional on the latter binding to the TNF receptor. This means that peptides with an agonistic or antagonistic effect compete with rhu-TNF for binding to the TNF receptor on TNF-sensitive indicator cells (e.g. U937). The competitive receptor-binding test was carried out as follows:

1. 100 µl of medium containing various concentrations of the peptide to be tested and of rhu-TNF (= control) were pipetted into the reaction vessels. The medium comprised 500 ml of PBS (Boehringer Mannheim), 10 ml of heat-inactivated (56°C, 30 min) FCS and 100 mg of sodium azide.

2. Subsequently, 100 µl of medium containing 1 ng of ^{125}I-labeled rhu-TNF (Bolton lactoperoxidase method) were placed in the reaction vessels and mixed. The non-specific binding (NSB) was determined by mixing in the reaction vessels the ^{125}I-labeled rhu-TNF (1 ng of ^{125}I-rhu-TNF in 100 µl of medium) with a 200-fold excess of unlabeled rhu-TNF (200 ng of rhu-TNF in 100 µl of medium).

3. Then 100 µl of medium containing 2 x 10^6 U937 cells (human) were pipetted into the reaction

vessels and mixed. The reaction vessels (test volume 300 μl) were incubated at 0°C for 90 min. The reaction mixtures were remixed after 45 min.

4. After the incubation the cells were centrifuged at 1800 rpm and 4°C for 5 min, washed 3 times with medium and transferred quantitatively into counting vials, and the cell-bound radioactivity was determined in a Clini gamma counter 1272 (LKB Wallac).

5. After the measurements had been corrected for the non-specific binding, the 50% competition value was defined by relation to the overall binding, and the sample concentration which led to 50% competition of ^{125}I-rhu-TNF binding at the ^{125}I-rhu-TNF concentration used was calculated as the competitive activity of the sample tested.

The Examples which follow are intended to explain the invention in more detail. The proteinogenous amino acids are abbreviated in the Examples using the conventional three-letter code. Other meanings are:
Aad = α-aminoadipic acid, Abs = 4-aminobutyric acid, Ac = acetic acid, Aoc = 8-aminooctanoic acid, Ape = 5-aminopentanoic acid, Hcy = homocysteine, Hly = homolysine, Orn = ornithine, Dap = 2,3-diaminopropionic acid.

A. General procedure

I. The peptides claimed in claim 1 were synthesized using standard methods of solid-phase peptide synthesis in a completely automatic model 430A peptide synthesizer from APPLIED BIOSYSTEMS. The apparatus uses different synthesis cycles for the Boc and Fmoc protective group techniques.

a) Synthesis cycle for the Boc protective group technique

1.	30% trifluoroacetic acid in DCM	1 x 3 min
2.	50% trifluoroacetic acid in DCM	1 x 17 min
3.	DCM washing	5 x 1 min
4.	5% diisopropylethylamine in DCM	1 x 1 min
5.	5% diisopropylethylamine in NMP	1 x 1 min
6.	NMP washing	5 x 1 min
7.	Addition of preactivated protected amino acid (activation by 1 equivalent of DCC and 1 equivalent of HOBt in NMP/DCM); peptide coupling (1st part)	1 x 30 min
8.	Addition of DMSO to the reaction mixture until it contains 20% DMSO by volume	
9.	Peptide coupling (2nd part)	1 x 16 min
10.	Addition of 3.8 equivalents of diisopropylethylamine to the reaction mixture	
11.	Peptide coupling (3rd part)	1 x 7 min
12.	DCM washing	3 x 1 min
13.	If reaction is incomplete, repetition of coupling (return to 5.)	
14.	10% acetic anhydride, 5% diisopropylethylamine in DCM	1 x 2 min
15.	10% acetic anhydride in DCM	1 x 4 min
16.	DCM washing	4 x 1 min
17	Return to 1.	

b) Synthesis cycle for the Fmoc protective group technique

1.	NMP washing	1 x 1 min
2.	20% piperidine in NMP	1 x 4 min
3.	20% piperidine in NMP	1 x 16 min
4.	NMP washing	5 x 1 min
5.	Addition of preactivated protected amino acid (activation by 1 equivalent of DCC and 1 equivalent of HOBt in NMP/DCM); peptide coupling	1 x 61 min
6.	NMP washing	3 x 1 min

7. If reaction is incomplete, repetition of coupling (return to 5.)
8. 10% acetic anhydride in NMP 1 x 8 min
9. NMP washing 3 x 1 min
10. Return to 2.

II. Working up of peptide-resins obtained as in Ia

The peptide-resin obtained as in Ia was dried under reduced pressure and transferred into a reaction vessel of a Teflon HF apparatus (from PENINSULA). Addition of a scavenger, preferably anisole (1 ml/g of resin), and of a thiol, in the case of tryptophan-containing peptides, to remove the indole formyl group, preferably ethanedithiol (0.5 ml/g of resin), was followed by condensation in of hydrogen fluoride (10 ml/g of resin) while cooling with liquid N_2. The mixture was allowed to warm to 0°C, and was stirred at this temperature for 45 min. The hydrogen fluoride was then stripped off under reduced pressure and the residue was washed with ethyl acetate in order to remove remaining scavenger. The peptide was extracted with 30% strength acetic acid and filtered, and the filtrate was freeze-dried.

To prepare peptide hydrazides, the peptide-resin (Pam- or Merrifield resin) was suspended in DMF (15 ml/g of resin), hydrazine hydrate (20 equivalents) was added, and the mixture was stirred at room temperature for 2 days. To work up, the resin was filtered off and the filtrate was evaporated to dryness. The residue was crystallized from DMF/Et_2O or MeOH/Et_2O.

III. Working up of the peptide-resins obtained as in Ib

The peptide-resin obtained as in Ib was dried under reduced pressure and subsequently subjected to one of the following cleavage procedures, depending on the amino acid composition (Wade, Tregear, Howard

Florey Fmoc-Workshop Manual, Melbourne 1985).

Peptide containing			Cleavage conditions		
Arg(Mtr)	Met	Trp	TFA	Scavenger	Reaction Time
no	no	no	95%	5% H_2O	1.5 h
yes	no	no	95%	5% thioanisole	≥ 3 h
no	yes	no	95%	5% ethyl methyl sulfide	1.5 h
no	no	yes	95%	5% ethanedithiol/ anisole (1:3)	1.5 h
no	yes	yes	95%	5% ethanedithiol/ anisole/ethyl methyl sulfide (1:3:1)	1.5 h
yes	yes	yes	93%	7% ethanedithiol/ anisole/ethyl methyl sulfide (1:3:3)	≥ 3 h

The suspension of the peptide-resin in the suitable TFA mixture was stirred at room temperature for the stated time and then the resin was filtered off and washed with TFA and with DCM. The filtrate and the washings were extensively concentrated, and the peptide was precipitated by addition of diethyl ether. The mixture was cooled in an ice bath, and the precipitate was filtered off, taken up in 30% acetic acid and freeze-dried.

IV. Purification and characterization of the peptides

Purification was by gel chromatography (SEPHADEX® G-10, G-15/10% HOAc; SEPHADEX® LH20/MeOH) and subsequent medium pressure chromatography (stationary phase: HD-SIL C-18, 20-45 μ, 100Å mobile phase: gradient with A = 0.1% TFA/MeOH, B = 0.1% TFA/H_2O).

The purity of the final products was determined by analytical HPLC (stationary phase: 100 x 2.1 mm VYDAC C-18, 5 μ, 300 Å; mobile phase = CH_3CN/H_2O gradient buffered with 0.1% TFA, 40°C). Characterization was by means of amino acid analysis and fast atom bombardment mass spectrometry.

B. Specific procedures

EXAMPLE 1

$H-Ala-Asn-Gly-Val-Glu-NH_2$

1.28 g of Boc-Glu(OBzl)-MBHA-resin (substitution 0.39 mmol/g), corresponding to a batch size of 0.5 mmol, were reacted as in AIa with 2 mmol each of

Boc-Val-OH
Boc-Gly-OH
Boc-Asn-OH
Boc-Ala-OH.

After the synthesis was complete, the peptide-resin underwent N-terminal deprotection (steps 1-3 as in AIa) and subsequent drying under reduced pressure; the yield was 1.35 g.

0.7 g of the resin obtained in this way was subjected to HF cleavage as in AII. The crude product (81 mg) was purified by gel filtration (SEPHADEX® G-10) and medium pressure chromatography (cf. AIV; 40-60% A; 0.25% min^{-1}).

EXAMPLE 2

Ac-Leu-Ala-Asn-Gly-Val-Glu-OH

0.46 g of Fmoc-Glu(OtBu)-p-alkoxybenzyl alcohol-resin (substitution 0.55 mmol/g) corresponding to a batch size of 0.25 mmol, was reacted as in AIb with 1 mmol each of

Fmoc-Val-OH
Fmoc-Gly-OH
Fmoc-Asn-OH
Fmoc-Ala-OH
Fmoc-Leu-OH

After the synthesis was complete, the N terminus was acetylated (steps 2-4 and 8-9 as in AIb). The resulting peptide-resin was dried under reduced pressure; the yield was 0.54 g.

The crude peptide (129 mg) obtained after TFA cleavage as in AIII was purified by gel filtration (SEPHADEX® G-10) and medium pressure chromatography (cf. AIV; 40-60% A; 0.25% min^{-1}). 67 mg of pure product were obtained.

The following were prepared in a similar manner to Examples 1 and 2:

3. H-Ala-Asn-Gly-Val-Glu-OH
4. Ac-Ala-Asn-Gly-Val-Glu-OH
5. Ac-Ala-Asn-Gly-Val-Glu-NH_2
6. H-Leu-Ala-Asn-Gly-Val-Glu-OH
7. H-Leu-Ala-Asn-Gly-Val-Glu-NH_2
8. Ac-Leu-Ala-Asn-Gly-Val-Glu-NH_2
9. H-Leu-Ala-Asn-Gly-Val-Glu-Leu-OH
10 Ac-Leu-Ala-Asn-Gly-Val-Glu-Leu-OH
11. H-Leu-Ala-Asn-Gly-Val-Glu-Leu-NH_2
12. Ac-Leu-Ala-Asn-Gly-Val-Glu-Leu-NH_2
13. H-Leu-Leu-Ala-Asn-Gly-Val-Glu-Leu-Arg-OH
14. Ac-Leu-Leu-Ala-Asn-Gly-Val-Glu-Leu-Arg-OH
15. H-Leu-Leu-Ala-Asn-Gly-Val-Glu-Leu-Arg-NH_2
16. Ac-Leu-Leu-Ala-Asn-Gly-Val-Glu-Leu-Arg-NH_2
17. H-Ala-Leu-Leu-Ala-Asn-Gly-Val-Glu-Leu-Arg-OH
18. Ac-Ala-Leu-Leu-Ala-Asn-Gly-Val-Glu-Leu-Arg-NH_2
19. H-Leu-Ala-Asn-Gly-Phe-Glu-OH
20. Ac-Leu-Ala-Asn-Gly-Phe-Glu-NH_2
21. Ac-Leu-Ala-Asn-Gly-Met-Glu-Leu-NH_2
22. Ac-Leu-Leu-Ala-His-Gly-Val-Glu-Leu-Arg-NH_2
23. Ac-Ile-Ala-Asn-Gly-Val-Glu-NH_2

EXAMPLE 24

Ac-Cys-Ala-Asn-Gly-Val-Glu-Cys-NH_2

0.57 g of Boc-Cys(pMB)-MBHA-resin (substitution 0.86 mmol/g), corresponding to a batch size of 0.5 mmol, was reacted as in AIa with 2 mmol each of

Boc-Glu(OBzl)-OH	Boc-Asn-OH
Boc-Val-OH	Boc-Ala-OH
Boc-Gly-OH	Boc-Cys(pMB)-OH.

After the synthesis was complete, the N terminus was acetylated (steps 1-6 and 14-16 as in AIa).

The resulting peptide-resin was dried under reduced pressure; the yield was 0.98 g.

0.49 g of the resin obtained in this way was subjected to HF cleavage as in AII. The freeze-dried crude product was taken up in 2 l of 0.1% strength acetic acid, and the pH was then adjusted to 8.4 with aqueous ammonia. Under an argon atmosphere, 0.01 N $K_3[Fe(CN)_6]$ solution was slowly added dropwise until the yellowish-green color persisted for at least 15 min. The mixture was then stirred for 1 h and then acidified to pH 4.5 with glacial acetic acid, and 15 ml of an aqueous suspension of an anion exchanger (BIORAD® 3 x 4A, chloride form) were added. After 30 min, the ion exchanger resin was filtered off, and the filtrate was concentrated to 100 ml in a rotary evaporator and subsequently freeze-dried.

All the solvents used had previously been saturated with nitrogen in order to prevent any oxidation of the free cysteine residues.

The crude product was purified by gel chromatography (SEPHADEX® G-15). 87 mg of pure product were obtained.

The following can be prepared in a similar manner to Example 24 (Pam-resin was used to prepare the peptide

acids):

25. Ac-Cys-Asn-Gly-Val-Cys-NH_2
26. Ac-Cys-Ala-Asn-Gly-Val-Cys-NH_2
27. H-Cys-Ala-Asn-Gly-Val-Cys-OH
28. H-Cys-Ala-Asn-Gly-Val-Glu-Cys-OH
29. Ac-Cys-Ala-Asn-Gly-Val-Glu-Cys-OH
30. H-Cys-Ala-Asn-Gly-Val-Glu-Cys-NH_2
31. Ac-Cys-Asn-Gly-Val-Glu-Cys-NH_2
32. H-Hcy-Ala-Asn-Gly-Val-Glu-Cys-OH
33. Ac-Hcy-Ala-Asn-Gly-Val-Glu-Cys-OH
34. H-Hcy-Ala-Asn-Gly-Val-Glu-Cys-NH_2
35. Ac-Hcy-Ala-Asn-Gly-Val-Glu-Cys-NH_2
36. H-Cys-Ala-Asn-Gly-Val-Glu-Hcy-OH
37. Ac-Cys-Ala-Asn-Gly-Val-Glu-Hcy-OH
38. H-Cys-Ala-Asn-Gly-Val-Glu-Hcy-NH_2
39. Ac-Cys-Ala-Asn-Gly-Val-Glu-Hcy-NH_2
40. H-Hcy-Ala-Asn-Gly-Val-Glu-Hcy-OH
41. Ac-Hcy-Ala-Asn-Gly-Val-Glu-Hcy-OH
42. H-Hcy-Ala-Asn-Gly-Val-Glu-Hcy-NH_2
43. Ac-Hcy-Ala-Asn-Gly-Val-Glu-Hcy-NH_2
44. H-Cys-Leu-Ala-Asn-Gly-Val-Glu-Cys-OH
45. Ac-Cys-Leu-Ala-Asn-Gly-Val-Glu-Cys-NH_2
46. H-Hcy-Leu-Ala-Asn-Gly-Val-Glu-Cys-OH
47. Ac-Hcy-Leu-Ala-Asn-Gly-Val-Glu-Cys-NH_2
48. H-Cys-Ala-Asn-Gly-Val-Glu-Leu-Cys-OH
49. Ac-Cys-Ala-Asn-Gly-Val-Glu-Leu-Cys-NH_2
50. H-Hcy-Ala-Asn-Gly-Val-Glu-Leu-Cys-OH
51. Ac-Hcy-Ala-Asn-Gly-Val-Glu-Leu-Cys-NH_2

52. H-Cys-Leu-Ala-Asn-Gly-Val-Glu-Leu-Cys-OH

53. Ac-Cys-Leu-Ala-Asn-Gly-Val-Glu-Leu-Cys-NH_2

54. H-Hcy-Leu-Ala-Asn-Gly-Val-Glu-Leu-Cys-OH

55. Ac-Hcy-Leu-Ala-Asn-Gly-Val-Glu-Leu-Cys-NH_2

56. H-Cys-Leu-Ala-Asn-Gly-Val-Glu-Leu-Hcy-OH

57. Ac-Cys-Leu-Ala-Asn-Gly-Val-Glu-Leu-Hcy-NH_2

58. H-Hcy-Leu-Ala-Asn-Gly-Val-Glu-Leu-Hcy-OH

59. Ac-Hcy-Leu-Ala-Asn-Gly-Val-Glu-Leu-Hcy-NH_2

60. Ac-Asn-Ala-Leu-Hcy-Ala-Asn-Gly-Val-Glu-Cys-Arg-Asp-NH_2

61. Ac-Hcy-Ala-Asp-Gly-Val-Glu-Cys-NH_2

62. H-Hcy-Ala-Asn-Gly-Val-Glu-Hcy-OH

63. H-Hcy-Ala-His-Gly-Val-Glu-Cys-OH

64. H-Cys-Leu-Ala-Asn-Gly-Val-Asp-Leu-Cys-OH

65. H-Hcy-Ala-Asn-Gly-Val-Cys-OH

66. Ac-Hcy-Ala-Asn-Gly-Val-Cys-NH_2

67. H-Cys-Ala-Asn-Gly-Val-Hcy-OH

68. Ac-Cys-Ala-Asn-Gly-Val-Hcy-NH_2

69. H-Hcy-Ala-Asn-Gly-Val-Hcy-OH

70. Ac-Hcy-Ala-Asn-Gly-Val-Hcy-NH_2

EXAMPLE 71

Ac-Lys-Ala-Asn-Gly-Val-Glu-NH_2

0.53 g of Boc-Glu(OChx)-MBHA-resin (substitution 0.95 mmol/g), corresponding to a batch size of 0.5 mmol, was reacted as in AIa with 2 mmol each of

Boc-Val-OH
Boc-Gly-OH
Boc-Asn-OH
Boc-Ala-OH
Boc-Lys(Cl-Z-)-OH

After the synthesis was complete, the N terminus was acetylated (steps 1-6 and 14-16 as in AIa). The resulting peptide-resin was dried under reduced pressure; the yield was 0.86 g.

The crude product (272 mg) obtained after HF cleavage as in AII was dissolved in 380 ml of degassed DMF, and 0.53 ml of triethylamine and, at -25°C, 0.54 ml of diphenylphosphoryl azide were added. The mixture was stirred at -25°C for 2 h, stored at -25°C for 2 days, at 4°C for 2 days and at room temperature for 2 days and subsequently evaporated to dryness. The crude peptide was purified by gel chromatography (SEPHADEX® G-15) and subsequent medium pressure chromatography (cf. AIV; 5-25% A; 0.25% min^{-1}). 51 mg of pure product were obtained.

EXAMPLE 72

Ac-Lys-Ala-Asn-Gly-Val-Glu-Glu-NH_2

2 g of resin described by Breipohl et al. (from BACHEM), corresponding to a batch size of 1 mmol, was reacted as in AIb with 4 mmol each of

Fmoc-Glu(OtBu)-OH
Fmoc-Glu(OBzl)-OH
Fmoc-Val-OH
Fmoc-Gly-OH
Fmoc-Asn-OH
Fmoc-Ala-OH
Fmoc-Lys(Boc)-OH

After the synthesis was complete, the N terminus was acetylated (steps 2-4 and 8-9 as in AIb). The peptide-resin was dried under reduced pressure; yield 2.71 g.

480 mg of the crude product (872 mg) obtained after TFA cleavage as in AIII were dissolved in 500 ml of degassed DMF, and 0.67 ml of triethylamine and, at -25°C, 0.67 ml of diphenylphosphoryl azide were added.The mixture was

stirred at -25°C for 2 h, stored at -25°C for 2 days, at 4°C for 2 days and at room temperature for 2 days and subsequently evaporated to dryness. The crude peptide was purified by gel chromatography (SEPHADEX® LH 20) and the isolated monomer (103 mg) was deprotected with HF as in AII and purified by medium pressure chromatography (cf. AIV, 5-25% A, 0.25% min^{-1}). 68 mg of pure product were obtained.

EXAMPLE 73

H-Lys-Leu-Ala-Asn-Gly-Val-Glu-Glu-OH

1.04 g of Fmoc-Glu(OtBu)-Merrifield resin (substitution 0.48 mmol/g), corresponding to a batch size of 0.5 mmol, were reacted as in AIb with 2 mmol each of

Fmoc-Glu(OBzl)-OH	Fmoc-Asn-OH	Fmoc-Lys(Boc)-OH
Fmoc-Val-OH	Fmoc-Ala-OH	
Fmoc-Gly-OH	Fmoc-Leu-OH	

The t-butyl and Boc protective groups were subsequently cleaved off (steps 1-6 as in AIa). The cyclization on the resin took place in NMP with the addition of 0.8g g of BOP and 0.87 ml of diisopropylethylamine (24 h). The peptide-resin underwent N-terminal deprotection (steps 2-4 as in AIb) and drying under reduced pressure. The yield was 1.35 g.

The crude product obtained after HF cleavage as in AII was purified by gel filtration (Sephadex® G-15) and medium pressure chromatography (cf. AIV; 10-30% A; 0.25% min^{-1}). 24 mg of pure product were obtained.

The following can be prepared in a similar manner to Examples 71, 72 and 73:

74. Ac-Aad-Asn-Gly-Val-Lys-NH_2

75. H-Glu-Ala-Asn-Gly-Val-Lys-OH

76. Ac-Glu-Ala-Asn-Gly-Val-Lys-NH_2

77. Ac-Glu-Ala-Asn-Gly-Val-Lys-OH

78. Ac-Lys-Asn-Gly-Val-Glu-NH_2

79. Ac-Lys-Asn-Gly-Val-Glu-OH

80. Ac-Glu-Ala-Asn-Gly-Val-Glu-Hly-NH_2

81. Ac-Asp-Ala-Asn-Gly-Val-Glu-Lys-NH_2

82. Ac-Asp-Ala-Asn-Gly-Val-Glu-Lys-OH

83. Ac-Asp-Ala-Asn-Gly-Val-Glu-Hly-NH_2

84. Ac-Orn-Ala-Asn-Gly-Val-Glu-Asp-NH_2

85. Ac-Lys-Ala-Asn-Gly-Val-Glu-OH

86. Ac-Lys-Ala-Asn-Gly-Val-Glu-Asp-NH_2

87. Ac-Lys-Ala-Asn-Gly-Val-Glu-Glu-OH

88. Ac-Lys-Ala-Asn-Gly-Val-Glu-Aad-NH_2

89. Ac-Hly-Ala-Asn-Gly-Val-Glu-Asp-NH_2

90. Ac-Aad-Ala-Asn-Gly-Val-Glu-Hly-NH_2

91. Ac-Aad-Ala-Asn-Gly-Val-Glu-Lys-NH_2

92. Ac-Lys-Ala-Asn-Gly-Val-Glu-NH_2

93. Ac-Glu-Ala-Asn-Gly-Val-Glu-Leu-Lys-NH_2

94. Ac-Lys-Leu-Ala-Asn-Gly-Val-Glu-Glu-NH_2

95. Ac-Glu-Ala-Asn-Gly-Val-Glu-Leu-Lys-NH_2

96. Ac-Lys-Ala-Asn-Gly-Val-Glu-Leu-Glu-NH_2

97. H-Asn-Ala-Leu-Glu-Ala-Asn-Gly-Val-Lys-Leu-NH_2

98. Ac-Glu-Gly-Asn-Gly-Val-Lys-NH_2

99. Ac-Ala-Leu-Glu-Ala-Asn-Gly-Val-Glu-Lys-Arg-Asp-NH_2

100. Ac-Asp-Ala-His-Gly-Val-Glu-Lys-NH_2

101. Ac-Lys-Ala-Asn-Gly-Phe-Glu-Glu-NH_2

102. Ac-Glu-Ala-Asn-Gly-Val-Glu-Lys-NH_2

EXAMPLE 103

Leu-Ala-Asn-Gly-Val-Glu-Leu-Abs

1.47 g of Fmoc-Gly-p-alkoxybenzyl alcohol-resin (substitution 0.68 mmol/g), corresponding to a batch size of 1 mmol, were reacted as in AIb with 4 mmol each of

Fmoc-Asn-OH
Fmoc-Ala-OH
Fmoc-Leu-OH
Fmoc-Abs-OH
Fmoc-Leu-OH
Fmoc-Glu(OBzl)-OH
Fmoc-Val-OH

After the synthesis was complete, the peptide-resin underwent N-terminal deprotection (steps 2-4 as in AIb) and subsequent drying under reduced pressure. The yield was 1.35 g.

200 mg of the crude peptide obtained after TFA cleavage as in AIII were dissolved in 200 ml of degassed DMF. 84 mg of $NaHCO_3$ and 264 mg of BOP were added and then the mixture was stirred at room temperature overnight. It was then evaporated to dryness, and the crude peptide was purified by gel chromatography (SEPHADEX® LH 20). The isolated monomer (74 mg) was deprotected with HF as in AII. 62 mg of pure product were obtained.

The following can be prepared in a similar manner to Example 103:

104. Ala-Asn-Gly-Val-Glu

105. Leu-Ala-Asn-Gly-Val-Glu-Leu-Arg

106. Aoc-Ala-Asn-Gly-Val-Glu

107. Ala-Asn-Gly-Val-Glu-Leu-Ape

108. Leu-Ala-Asn-Gly-Val-Glu-Leu-Ape

109. Leu-Ser-Asn-Gly-Val-Glu

110. Leu-Ala-His-Gly-Val-Glu-Leu-Arg

111. Ala-Asn-Gly-Met-Glu-Leu-Ape

112. Leu-Ala-Asn-Gly-Val-Glu .

We claim:-

1. A peptide of the formula I

$$X\text{-}A\text{-}Gly\text{-}B\text{-}Y \qquad I$$

where

A is Asn, Asp or His,

B is Val, Met or Phe,

X is G-NH-CHM-CO-, G-NH-CHM-CO-W-, G-R-NH-CHM-CO- or G--R-NH-CHM-CO W- and

Y is -Z,-NH-CHQ-CO-Z, -V-NH-CHQ-CO-Z, -NH-CHQ-CO-U-Z or -V-NH-CHQ-CO-U-Z

where, in X and Y,

G is hydrogen or an amino-protective group,

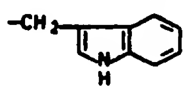
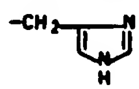
Z is OH or NH_2 or a carboxyl-protective group or

G and Z together are also a covalent bond or $-CO-(CH_2)_a-NH-$ where a is from 1 to 12,

R, U, V and W are peptide chains composed of 1-4 naturally occurring α-amino acids, and

M and Q are hydrogens or one of the following

$-CH(CH_3)_2$, $-CH(CH_3)-C_2H_5$, $-C_6H_5$, $-CH(OH)-CH_3$,

 ,  or $-(CH_2)_b-T$

(b being from 1 to 6 and T being hydrogen or OH, CH_3O, CH_3S, $(CH_3)_2CH$, C_6H_5, p-HO-C_6H_4, HS, H_2N, HO-CO, H_2N-CO, $H_2N-C(=NH)-NH$) or

M and Q together are a $-(CH_2)_c-S-S-(CH_2)_d$, $-(CH_2)_e-CO-NH-(CH_2)_f$ or $-(CH_2)_e-NH-CO-(CH_2)_g-NH-CO-(CH_2)_f-$ bridge (with c and d being from 1 to 4, e and f being from 1 to 6 and g being from 1 to 12),

as well as the salts thereof with physiologically tolerated acids.

2. A peptide as claimed in claim 1, where G is hydrogen or an amino-protective group and Z is hydroxyl or amino or a carboxyl-protective group, and M and Q are not connected together.

3. A peptide as claimed in claim 1, where G is hydrogen or an amino-protective group and Z is hydroxyl or amino or a carboxyl-protective group, and M and Q together are a $-(CH_2)_c-S-S-(CH_2)_d-$ bridge.

4. A peptide as claimed in claim 1, where G is hydrogen or an amino-protective group and Z is hydroxyl or amino or a carboxyl-protective group, and M and Q together are $-(CH_2)_e-NH-CO-(CH_2)_f-$ or $-(CH_2)_e-NH-CO-(CH_2)_g-NH-CO-(CH_2)_f$.

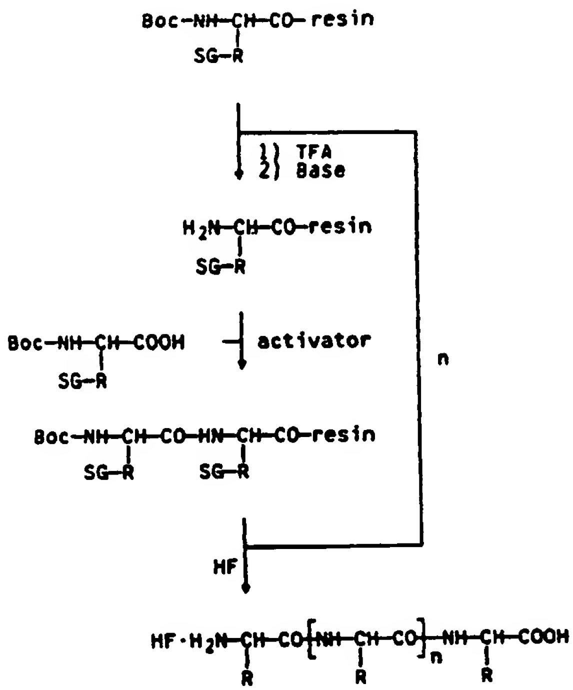
5. A peptide as claimed in claim 1, where G + Z together are a covalent bond or $-CO-(CH_2)_a-NH-$.

6. A peptide as claimed in claims 1 to 5 for use for controlling diseases.

7. The use of the peptides as claimed in claims 1 to 5 for controlling neoplastic diseases and autoimmune diseases as well as for control-ling and preventing infections, inflammations and transplant rejection reactions.

8. A process for the preparation of a peptide as claimed in claims 1 to 5, which comprises preparation thereof using conventional methods of peptide chemistry.

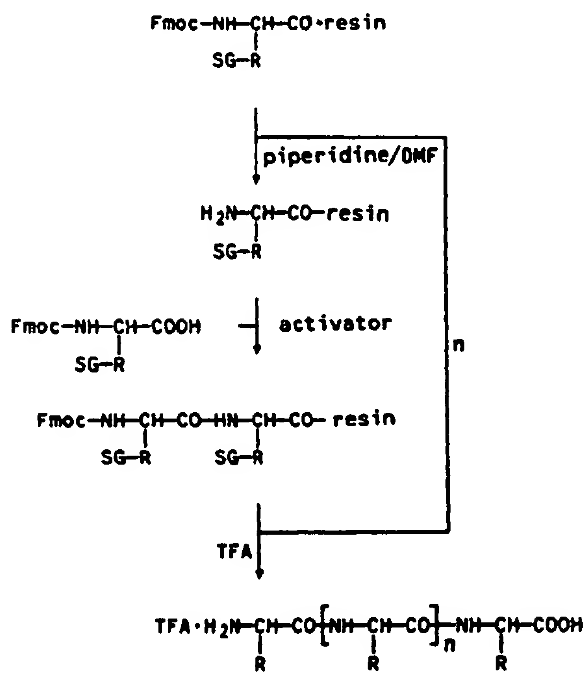
Fig. 1: The Boc protective group technique on a polymeric support



Boc = t-butyloxycarbonyl protective group
SG = side-chain protective group
R = amino acid side-chain

Patent Agents.

Fig. 2: The Fmoc protective group technique on a polymeric support



Fmoc = 9-fluorenylmethoxycarbonyl protective group
SG = side-chain protective group
R = amino acid side-chain.

Patent Agents.

ABSTRACT OF THE DISCLOSURE:

Peptides of the formula

X-A-Gly-B-Y,

where A, B, X and Y are defined in the description, and the preparation thereof are described. The novel peptides are suitable for controlling diseases.